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Acute Cytotoxic Allogeneic Histoincompatibility Reactions Involving Gray Cells in the Marine Sponge, *Callyspongia diffusa*

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Abstract. A variety of procedures were used in a study of the histoincompatibility reactions of *Callyspongia diffusa*. Rejection reactions as traditionally tested between laterally apposed intact fingers cut from two different sponges require about a week of contact to exhibit cytotoxicity. In a miniaturized assay involving reactions between small pieces of tissue snipped from sponges with scissors and pushed together on an insect pin, cytotoxicity is evident within 48 hours of contact. Reactions of cells dissociated by divalent cation removal and allowed to reaggregate in seawater were also studied. Aggregates produced from allogeneic mixtures of cells from two individuals were killed by internal cytotoxic reactions within 36 hours of the initiation of aggregation. After only one hour of aggregation, aggregates from allogeneic mixtures were significantly smaller than aggregates of cells from a single individual. This rapid slowing of aggregation is the earliest response to allogeneic contact that we noted and does not appear to reflect early cytotoxic processes. Apposition of an aggregate containing cells from one sponge to an aggregate containing cells from a second individual leads to mutual destruction. Aggregates harvested and apposed 4 hours after initiation of aggregation begin to show mutual cytotoxicity at 36 hours of contact. Aggregates placed in contact 48 hours after the initiation of aggregation exhibit cytotoxicity within 8 hours. These rapidly reacting 48-hour aggregates exhibit a pronounced accumulation of gray cells at the boundary of allogeneic contact by 8 hours. These results are interpreted as indicating at least five steps in the histoincompatibility reactions of *C. diffusa:* (1) recognition soon after allogeneic contact; (2) generation of signals that suppress cell aggregation and cell movement and attract gray cells to the boundary of contact; (3) acceleration of the sponge immune response—including the responsiveness of gray cells to accumulate at the boundary of allogeneic contact—by tissue trauma produced when the tissue is cut or dissociated it into individual cells; (4) arrival of gray cells at the boundary of allogeneic contact; and (5) initiation of cytotoxic processes.

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Introduction

The invertebrate immune system must protect the individual animal from a full range of parasitic and pathogenic threats (Ratcliffe *et al.*, 1985). For sessile species, individual-specific recognition may also prevent cellular parasitism between contacting individuals of the same species (Buss, 1987). Very specific, immune-like histoincompatibility reactions occur in every invertebrate phylum (Cooper *et al.*, 1992) including sponges, the simplest metazoans (Hildemann *et al.*, 1979). Because these reactions are usually very slow, and not well defined histologically, cells functioning like vertebrate T or B cells are not evident in any invertebrate. The idea that evolutionary precedents for specific recognition events characteristic of these vertebrate immune cells cannot be found in invertebrates is often expressed (Klein, 1989; Janeway, 1992; Smith and Davidson, 1992).

This laboratory has observed very rapid allogeneic recognition reactions involving the intimate participation of a specific cell type—gray cells—in the marine sponge *Microciona prolifera* (Humphreys, 1994). The rapidity and selectivity of these reactions have led us to focus again on the possibility of specific cellular recognition in the immune reactions of invertebrates (Humphreys and

Reinherz, 1994). Following Burnet's (1971) original suggestion, we have emphasized direct, specific, self recognition as the guiding principle for invertebrate immunity. Conceptually, if invertebrate immunocytes recognize individual self histocompatibility marker proteins, they can immediately, upon contact, recognize as nonself all cells that do not display the identical self-marker proteins. The ability of invertebrate immunocytes to recognize self could be likened to the ability of vertebrate T-cells to recognize self major histocompatibility complex (MHC) proteins. Just as vertebrate T-cells learn to recognize self MHC proteins through the process of positive selection, one can imagine that during differentiation, invertebrate immunocytes learn to recognize self proteins encoded by the specific alleles that the individual animal has inherited at a polymorphic locus (or loci) that encodes self marker proteins (Humphreys and Reinherz, 1994).

The tropical marine sponge *Callyspongia diffusa* was used in some of the first experiments on histoincompatibility in sponges (Hildemann *et al.*, 1979). Subsequently, graft rejection reactions have been described in a variety of sponge species and two distinct rejection modalities occur (Van de Vyver and Buscema, 1988). Some sponges—for example, *C. diffusa*—produce a nonspecific cytotoxic reaction that kills all cells within a few millimeters of the boundary of foreign contact (Hildemann *et al.*, 1980). Other species, such as *M. prolifera*, carry out an encapsulation-like process and produce a barrier, often containing collagen, between the contacting tissues (Humphreys, 1994). Our observations on *M. prolifera* suggest immediate cellular recognition upon allogeneic contact with the release of a chemokine that attracts the gray cells to accumulate in the zone of contact and organize the formation of the collagen layer (Humphreys, 1994). Although reactions of other species of sponges have been examined in considerable histological detail (Van de Vyver and Buscema, 1988; Smith and Hildemann, 1986a, b), few other clues concerning the cellular and mechanistic bases of recognition or rejection reactions have emerged.

Encouraged by a report that mixed aggregates of dissociated cells from two individuals of *C. diffusa* undergo a rapid rejection reaction which causes self annihilation of the aggregates within two days (Johnston, 1988), we began to explore the possibility of more rapid assays of recognition in this species. In fact, we describe cellular assays that can detect recognition reactions within an hour of allogeneic contact. We have also discovered acceleration and maturation processes that are components of the reacting immune system. In addition we have described gray cells in *C. diffusa* and have shown that recognition signals cause them to accumulate at the zone of allogeneic contact in association with the cytotoxic process. These results provide a beginning for productive cellular level analysis of allogeneic recognition in this species.

Materials and Methods

Sponges

The brilliant purple sponge *Callyspongia diffusa* (Ridley) was collected from the shallow reefs around Coconut Island, Kaneohe Bay, Oahu, Hawaii, and maintained in the running seawater tanks at the Kewalo Marine Laboratory. Sponges of 10 to 50 grams or more survive and continue to grow slowly for six months to one year in these tanks in spite of the excision, from time to time, of small portions of their tissue for experiments.

Histocompatibility assays

In addition to the original grafting system described for *C. diffusa* (Hildemann *et al.*, 1979) in which two intact fingers, typically several centimeters long, are tied together on a 2″ × 3″ microscope slide maintained in seawater, we have developed a miniaturized assay. Two small pieces of tissue a few mm in dimensions, snipped from the sponge with scissors, are pushed together on the end of a #0 stainless steel insect pin that is incubated standing in 3 ml of filtered seawater in a well of a 24-well tissue culture plate (Falcon type 3047) shaking at 50 rpm on a rotatory shaker. The seawater is changed daily.

We also assayed the histoincompatibility reactions that occur within aggregates of dissociated cells produced with mixtures of cells from two different sponges, as well as the reactions that occur between fully developed aggregates apposed in allogeneic pairs. The details of these assays follow a description of cell dissociation and reaggregation.

Cell dissociation and reaggregation

Sponge tissue (5 to 10 mm of a finger of the sponge, about 0.5 gram), is washed four times, 10 minutes per wash, with 10 ml per gram of tissue of calcium and magnesium free seawater (CMF-SW) (Humphreys, 1963) at room temperature, 22–25°C (lowering the temperature to 4°C kills *C. diffusa* cells). The washed tissue is then squeezed repeatedly in a bag of 100 μm Nitex mesh to disperse the cells from the matrix into 10 ml CMF-SW per gram of tissue. The cell concentration of the suspension typically ranges from 10 to 20 $\times 10^6$ cells per ml. The viability of the cells is checked by dye exclusion, either with 1% trypan blue in CMF-SW or the live/dead viability/cytotoxicity kit of Molecular Probes, Inc. (L-3224). Freshly dissociated cells routinely have a viability greater than 95%. The dissociated cells are centrifuged at 1500 rpm for 1½ min at 15°C (Sorvall Rt6000B Refrigerated Centrifuge), resuspended at a concentration of 10 $\times 10^6$ cells/ml in 0.45 μm Millipore filtered SW supple-

mented with 0.01% bovine serum albumen (BSA-SW; the addition of the very dilute BSA to the SW seems to improve the viability and health of the cells and aggregates). To produce aggregates, the cell suspension is immediately dispensed in 0.4-ml aliquots into 16-mm diameter wells of 24-well tissue culture plates (Falcon type 3047). Aggregation is initiated by placing the multiwell plates on a rotatory platform shaker with a 3″ diameter of rotation (Henkart and Humphreys, 1970) shaking at 50 rpm. The BSA-SW is gently replaced at 8 hours and then every day. For time series determinations, replicate multiwell plates are started to provide an undisturbed sample for each time point determination. Aggregates are photographed with direct illumination under a Wild M5 binocular microscope using Kodak Ektachrome film.

Histoincompatibility reactions in aggregates

To examine the reactions of allogeneic mixtures of cells, aggregates containing cells from two different sponges are produced. Two 0.2-ml aliquots of dissociated cell suspension from each of two sponges are mixed in the same aggregation well of a multiwell plate, which is then placed on the rotatory shaker.

The reactions of pairs of aggregates are examined by manually apposing two aggregates, each made up of cells of only one sponge. Aggregates are selected individually from the aggregation wells using a micropipette with an 0.5 mm bore under a binocular microscope. Aggregates 0.2 to 0.3 mm in diameter are selected and paired in the bottom of a well of a 96-well, U-bottom Micro Test III flexible Assay Plate (Falcon type 3911) with 0.1 ml of filtered seawater. For allogeneic reactions, an aggregate from one aggregation well containing cells of only one sponge is apposed to an aggregate from a different aggregation well containing only cells from a different sponge.

Gray cell fractionation

Gray cells are purified by centrifuging a preparation of dissociated cells in CMF-SW layered over a cushion of 8% Ficoll, 16% sodium diatrizoate in CMF-SW at 3000 rpm in an IEC refrigerated centrifuge at 15°C for 15 minutes. The pellet is resuspended in CMF-SW and consists of about 50–70% gray cells (See Fig. 6A and 6B).

Results

Tissue grafting

The original histocompatibility experiments with *C. diffusa* were replicated by tying two fingers cut from different sponge individuals side by side on a glass slide maintained in running seawater (Hildemann *et al.*, 1979). As described previously, the fingers adhere strongly for several days without discernible differences between autogeneic and allogeneic contacts. At about 6 days, depending somewhat on temperature (Johnston *et al.*, 1981), the organization of the cells along the zone of contact changes visibly, and 24 to 36 hours later cellular degeneration becomes apparent along the boundary of contact. As already analyzed extensively (Jokiel *et al.*, 1982), certain allogeneic combinations of specific individuals reproducibly react weakly or slowly, presumably due to genetic similarity of individual sponges in these populations. For the results presented here, reactive sponge pairs were used in all experiments.

We miniaturized the histocompatibility assay by examining the reactions of two small pieces of tissue pressed together on an insect pin. These pieces adhere rapidly in both autogeneic and allogeneic combinations (Fig. 1). The former heal together and remain healthy indefinitely. The latter usually exhibit degeneration along the zone of contact beginning by 48 hours. Figure 1 shows insect pin assays of the three autogeneic and the three allogeneic pairings possible between three individual sponges after 72 hours of contact, when degeneration of tissue is very evident along the boundary of contact in the allogeneic pairs. An interesting feature of the reacting tissue in these insect pin assays, evident on pins C and D in Figure 1, is the migration of cells onto the pin away from the area of cytotoxic reactions. Such emigration is not observed in non-reacting tissue. All living cells are usually eliminated from the original sponge matrix in these miniaturized grafts by 4 days. Under this protocol—involving both wounding of the contact surfaces and trauma to the sponge tissue by the crushing action of the scissors during the process of cutting the small pieces—allogeneic reactions are significantly more rapid with cell degeneration evident by 48 hours. This may be contrasted with reactions in about 7 days for laterally apposed complete fingers that have not been traumatized by cutting in the zone of contacting tissue (Hildemann *et al.*, 1980).

Cell reaggregation

Following the observations of Johnston (1988) on *C. diffusa*, we examined histoincompatibility reactions of allogeneic mixtures of reaggregated cells. We confirmed that cells, mechanically dissociated in seawater (SW) and allowed to settle on glass or plastic, aggregate and differentiate into small functional sponges within a few days when they are derived from one individual. In contrast, allogeneic mixtures of cells from two sponges die within 48 hours. We also examined these reactions with cells that had been dissociated by the removal of divalent cations (Humphreys, 1963), returned to complete SW, and reaggregated in suspension in wells of a multiwell plate on a rotatory shaker. Similar killing of mixed aggregates occurs under these conditions.

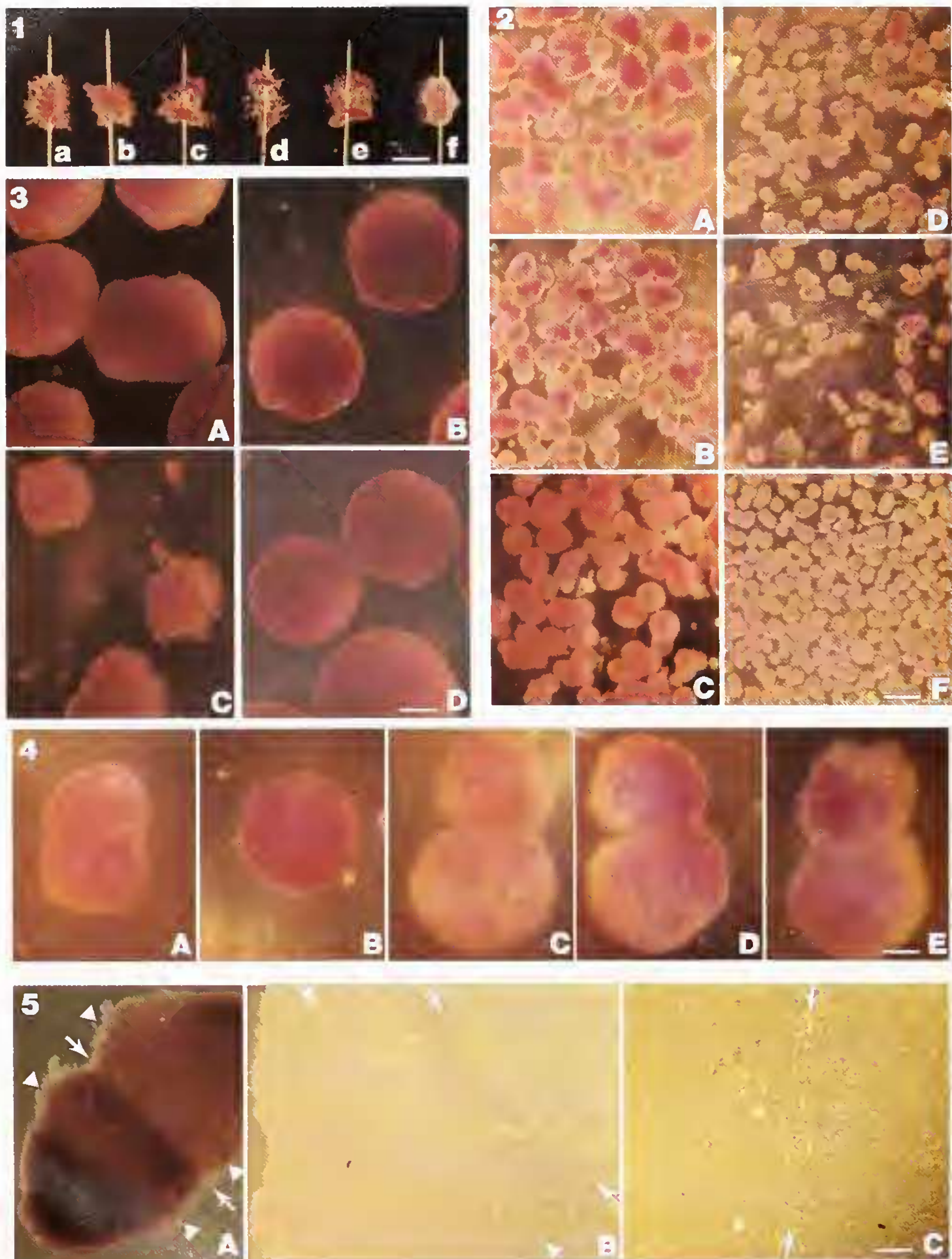

Figure 1. Histoincompatibility assay. The three autograft and the three allograft combinations possible between three individual sponges are shown 72 hours after apposition of pieces of tissue on insect pins. Two apposed pieces of tissue from the same sponge remain healthy and heal together (a, b, f). Two pieces of tissue from different sponge individuals react and create a zone of cell death exposing the skeletal matrix along the zone of allogeneic contact (c, d, e). Bar equals 0.4 cm.

Figure 2. Aggregates from dissociated cells from each of three individual sponges. Aggregates of pure populations of cells from the three sponges (A, B, C), and the three possible pair-wise allogeneic mixtures of dissociated cells from the three sponges (D, E, F), 2 hours after the initiation of aggregation in suspension shaker cultures. The aggregates of mixed cells from two individuals in D, E, and F are significantly smaller than aggregates from individual pure cell aggregates in A, B and C. Bar equals 0.5 mm.

Figure 3. Cytotoxic histoincompatibility reactions in aggregates containing an allogeneic mixture of dissociated cells derived from two different sponges. A. A lumpy surface at 20 hours of aggregation is the first evidence of cytotoxic reactions in mixed aggregates. B. By 21 hours the roughened appearance of the aggregate surface has become more pronounced. C. By 26 hours the mixed aggregates have begun to fall apart into smaller cell clumps which soon disintegrate completely. D. Aggregates of cells derived from one individual remain smoothly spherical as shown after 82 hours of aggregation. Bar equals 0.2 mm.

Table I

Average diameter of the ten largest aggregates in shaker wells after 2 hours of aggregation

Cells from sponge	Aggregate size (mm)
T	0.42 ± 0.07
W	0.40 ± 0.07
X	0.34 ± 0.05
T + W	0.24 ± 0.03
T + X	0.23 ± 0.02
W + X	0.23 ± 0.02

Suspensions of dissociated cells from an individual sponge adhere rapidly, forming ragged clumps within minutes. These aggregates in suspension become smooth and rounded (Fig. 2A, B, C) and attain maximum dimensions of 0.3 to 0.5 mm (Table I) by 2 hours. Although further aggregation after 2 hours is limited by the shearing forces in the shaker wells, these aggregates remain very adhesive and will adhere to each other or to the substrate within minutes if shaking is discontinued. When two spherical aggregates adhere but remain in suspension and shaking is reinitiated, they rapidly round up into a single larger spherical aggregate. If aggregates are allowed to attach to the substrate, they flatten and differentiate into functional sponges in 48 to 72 hours. Aggregates of cells from a single individual remain viable and healthy in suspension for days (Fig. 3D).

Allogeneic mixtures of cells in SW on a rotatory shaker also adhere rapidly, but aggregation slows appreciably in the first hour. By two hours (Fig. 2D, E, F), when they have attained maximum diameter, they are 0.20 to 0.25 mm (Table I), which is about 0.6 the diameter and one fifth the cell number of the aggregates formed from unmixed cells. The slowing of aggregation is not due to obvious cytotoxic processes. The viability of the cells, examined by dye exclusion after dissociating the aggregates with EGTA, remained over 95% for up to 20 hours. If shaking is stopped, these allogeneic aggregates adhere to each other but do not tend to round up into a single sphere. Instead, they maintain their original aggregate identity within the clump. These aggregates, when allowed to attach to the substrate, never flatten and differentiate. This rapid inhibition of cell aggregation and remodeling of aggregates provides an interesting early indication that allogeneic recognition has occurred.

At about 24 hours, cell degeneration becomes manifest in the allogeneic mixed aggregates maintained in suspension. The first indication of change is a lumpy appearance of the previously smooth outer surface of the aggregates (compare Fig. 3A with Fig. 3D). In the following 1–2 hours, the surfaces of the aggregates become rough and irregular (Fig. 3B). The aggregates soon begin to break open and fall apart into smaller clumps as shown at 26 hours (Fig 3C). During this latter period the percentage of cells failing to exclude dye also begins to rise. Over the next few hours, as the remaining clumps of cells fall apart, the cells lyse.

Aggregate pairs

The ability of fully formed aggregates of dissociated cells from one sponge to carry out a histocompatibility reaction when contacted by an aggregate of cells from another sponge (Humphreys, 1994) was examined in *C. diffusa* by apposing round aggregates formed in suspension. When two aggregates of cells from the same individual are pushed together in SW in a conical well of a microtiter plate, they adhere within minutes, undergo substantial healing together by 2 hours (Fig. 4A), and form a single round aggregate by 4–6 hours (Fig. 4B). This remodeling of the aggregates from two spheres into one sphere reflects the tendency of rapidly migrating, adhesive cells to maximize adhesions as they move rapidly over each other (Steinberg, 1963).

If an aggregate of cells from one individual sponge is placed in allogeneic contact with an aggregate of cells from a different sponge, they also adhere within minutes but, as shown at 2 hours in Figure 4C, do not then lose their separate aggregate identities. Even though they continue to adhere tightly, they remain as two distinct

Figure 4. Allogeneic histoincompatibility reactions between apposed 24-hour aggregates of dissociated cells. A and B. Pairs of aggregates from the same sponge heal together rapidly as shown after two hours in A. By six hours in B the original two spherical aggregates have melded together to form one single, spherical aggregate. C, D, and E. Apposed aggregates from two different sponges adhere, but do not heal together, after 2, 6, or 12 hours of contact (C, D, and E, respectively). By 12 hours of contact, the aggregates have become lumpy, evidencing the initiation of cytotoxic reactions. Bar equals 0.1 mm.

Figure 5. Migration of gray cells to the zone of allogeneic contact in apposed 48-hour aggregates from two individuals. A. After 6 hours of contact a zone of less pigmented, light refractive tissue (arrowheads) parallel to the boundary of allogeneic contact (arrows) is evident in an aggregate pair viewed and photographed with incident illumination under a binocular microscope. B. The aggregate shown in A, which has been compressed under a coverslip and viewed and photographed at low power with DIC optics. The light zone can be seen to represent the accumulation of refractive cells which, at this magnification, appear only as the brighter objects scattered among the otherwise purplish tissue. C. The refractive cells ultimately congregate at the boundary of allogeneic contact. Bar equals 0.1 mm.

groupings of cells as shown after 6 or 12 hours in Figure 4D and E. In Figure 4E the edges of the aggregates near the boundary of allogeneic contact have become rough. Over the next 6 to 8 hours the aggregates will break down into smaller clumps of cells that proceed to disintegrate completely. The failure of the cells within the two adherent aggregates to round up into one spherical aggregate suggests that cell movement has been suppressed by the histoincompatibility reactions.

We also discovered that mature aggregates harvested 48 hours after initiation of reaggregation react more quickly than newly formed aggregates. Aggregates collected 4 to 6 hours after the start of aggregation exhibit cytotoxic processes only after 36 hours of contact. Aggregates 24 hours old react more quickly (Fig. 4) and 48-hour aggregates initiate cytotoxic processes within 8 hours of apposition. Thus, the processes responsible for the cell-killing reactions mature and proceed more rapidly in the older aggregates.

Gray cells

Because gray cells are intimately involved in the histoincompatibility reactions of *M. prolifera* (Humphreys, 1994), we examined the gray cells of *C. diffusa*. Previous histological studies on *C. diffusa* (Smith and Hildemann, 1986a, b) failed to identify gray cells. However, an examination of CMF-SW-dissociated, living *C. diffusa* cells under Kohler or differential interference contrast (DIC) optics revealed a small but distinct population of large, multigranular cells resembling gray cells. These cells, concentrated by purification on a density gradient, are shown in Figure 6 A and B as photographed at high power with DIC optics. The characteristic feature of gray cells is the cytoplasm filled with densely packed, highly refractive, oblate granules. The gray cells do not contain the purple pigment evident in Figure 6A and B in the variety of cell types contaminating the gray cell preparation.

Gray cells were originally described in *M. prolifera* as appearing gray when viewed live under Kohler bright field microscopy (Wilson and Penney, 1930). Gray cells appear gray under Kohler optics because they are unpigmented, and their cytoplasm is completely filled with small granules of high refractive index that create an aura of darkness about the cell. Because the original designation of the gray cells in *M. prolifera* was based on their microscopic appearance, we refer to these corresponding cells in *C. diffusa* as gray cells. Gray cells represent about 1 to 3% of the total cells released from *C. diffusa* tissue by dissociation in CMF-SW.

In *M. prolifera*, gray cells accumulate at the boundary of allogeneic contact, creating a visible band in the reacting tissue (Humphreys, 1994). We examined reacting intact fingers, reacting pieces of tissue on insect pins, and reacting newly formed aggregates of *C. diffusa* to ascertain whether similar accumulations of gray cells occur at the boundary between reacting tissue in this species. Although there were suggestions in many experiments that gray cells were accumulating, we were unable to devise protocols that would yield striking and consistent accumulation of gray cells at the boundary of reaction

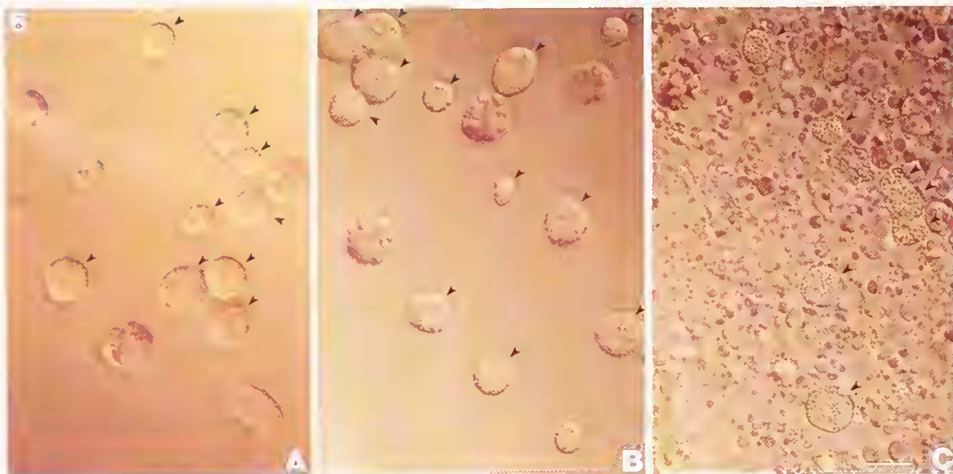

Figure 6. Gray Cells. A and B. Dissociated gray cells enriched by density gradient centrifugation C. Gray cells in a migrating band scattered among the purplish cells of a reacting 48-hour aggregate; 6 hours after the aggregates were apposed, they were compressed to 10 to 20 μm under a coverslip, examined, and photographed. Gray cells (marked by arrowheads) are bright, non-pigmented cells whose cytoplasm is packed with small oblate granules. The various non-gray cell types in the preparations can be seen to exhibit a diversity in number and type of cytoplsamic inclusions and the purple pigmentation characteristic of *C. diffusa* Observed at 100× with differential interference contrast optics. Bar equals 10 μm.

in these experimental settings. However, when 48-hour aggregates, which react in 8 hours, are apposed, a band of lighter colored tissue parallel to the boundary of contact became detectable within four to eight hours. A pair of reacting 48-hour aggregates, photographed 6 hours after pairing, exhibits such a band, and it seems to be converging on the boundary of allogeneic contact (Fig. 5A). If the adhering aggregates are greatly compressed under a cover slip until they are amenable to examination by DIC optics, an accumulation of gray cells is evident. A general view, taken with a 20× DIC objective, shows the aggregate pair from Figure 5A compressed to one fifth its original thickness (Fig. 5B). The brighter spots arrayed parallel to the boundary of allogeneic contact among the otherwise lightly purple cells represent gray cells. If the aggregates are compressed even more and observed with a 100× oil DIC objective, these brighter spots can be seen to be definitive gray cells with their densely packed oblate granules (Fig. 6C). One or two hours later, when the aggregates first exhibit signs of cytotoxic reactions, the gray cells are concentrated at the boundary of contact between the allogeneic cells as shown at low power in Figure 5C. We were unable to continue to find and identify the gray cells in the aggregates beyond this period during which the cytotoxic reactions begin. No accumulation of gray cells occurs in remodeling pairs of contacting aggregates of cells from the same sponge.

Discussion

We have studied the histoincompatibility reactions in the marine sponge *C. diffusa* and have developed novel modifications of this interesting experimental system that will help to elucidate the cellular and molecular mechanisms of immune recognition in the invertebrates. The histoincompatibility reactions in this species have been well studied (Hildemann *et al.*, 1979). Cytotoxic reactions between parabiosed intact fingers of two different sponges occur after a week and destroy a few millimeters of tissue along the boundary of allogeneic contact. These reactions have been followed by extensive histological examination (Smith and Hildemann 1986a, b; 1988) without a clear delineation of any specifically immune cells. The observations of Johnston (1988), that allogeneic mixtures of dissociated cells begin cytotoxic reactions within 48 hours, suggested to us that further examination of these more rapid reactions of dissociated cells might yield further insight into possible cellular mechanisms.

Our experiments present two new results that may be important for understanding the cell biology of recognition. We discovered an experimentally observable parameter, the rate of cell movement and aggregation, that changes within one hour of actual allogeneic contact, possibly reflecting the generation of a non-self recognition signal. This regulation of cell behavior can serve as a time- and possibly cell-proximal assay for the activation of the rejection reaction. It could prove useful for identifying the cells involved in recognition and signaling and for screening compounds, such as sugars, cyclosporin A, or monoclonal antibodies, which impinge directly on the early cellular events of recognition.

We have also established that *C. diffusa* has a population of gray cells that is specifically involved in the allogeneic rejection reaction. Sponges in general (Simpson, 1984), and *C. diffusa* specifically (Smith and Hildemann, 1986a, b; 1988), have fewer than a dozen cell types. The suggestion that gray cells, which are present in most sponge species (Boury-Esnault, 1977), may serve an immune function (Humphreys, 1994) provides insight into the nature of this cell type, whose function has otherwise been unknown (Simpson, 1984). Identifying a specific cell type found in many species of sponges as a possible immunocyte will provide focus for cellular and molecular studies of immune function in sponges.

The following aspects of our results will be discussed more fully: (1) the basis for the more rapid, 48-hour reactions of the tissue fragments on insect pins, and of allogeneic mixed cells in aggregates, relative to the one-week reactions of intact tissues; (2) the nature of the reactions that inhibit cell aggregation of the allogeneic cell mixtures; and (3) The processes occurring in newly formed, individual-specific aggregates that shorten the time to cytotoxic reactions from 36 hours of allogeneic contact to 8 hours and allow the rapid accumulation of gray cells at the allogeneic boundary.

48-hour reactions

When intact fingers of *C. diffusa* are apposed, cytotoxic reactions are not detectable until about one week later (Hildemann *et al.*, 1980, and our own experiments). In contrast, the cytotoxic reactions in the insect pin assay or within aggregates of dissociated cells begin in less than 48 hours. We propose that wounding or dissociation of the tissue quickly releases rate-limiting steps to the initiation of the sponge immune response, a process that requires several days when activated by allogeneic contact signals alone. The biological purpose for rate-limiting steps may be to delay as much as possible the nonspecific cell killing, which destroy the cells of the reacting animal as well as those of the foreign organism (Bigger *et al.*, 1981). Thus, the sponge seems to have mechanisms that respond slowly against foreign contacts when they minimally impinge on the sponge tissue, but can react vigorously to foreign contacts when tissue damage is involved. The more rapid second-set reactions that have been described in *C. diffusa* (Hildemann *et al.*, 1980) may be related to this acceleration. These authors noted that for about three weeks after undergoing a rejection reaction,

a finger of *C. diffusa* will react more rapidly to a second allogeneic contact.

Inhibition of aggregation

The inhibition of aggregation, which is manifested in an allogeneic mixture of dissociated cells within an hour of first contact, appears to be a specific response to non-self recognition upon allogeneic contact. We believe that aggregation slows because cell movement is suppressed, possibly by a chemokine released as part of the signaling of non-self recognition. In the experimental setting, a reduction in cell motility would slow the formation of larger cell clumps, because cell movement is required to stabilize nascent attachments between two cell clumps when they happen to make contact in the shaking suspensions. Instead of cells moving so that the initial adhesion spreads and becomes more stable, the two clumps adhere only at the point of initial contact and are ultimately sheared apart again by the motion of the liquid. Indeed, if shaking is stopped the allogeneic aggregates will adhere but do not tend to form a single sphere, a process requiring cell movement (Steinberg, 1963). Likewise the failure of two aggregates in allogeneic contact to round up into one spherical aggregate, even though they remain adherent, confirms that cell movement has been suppressed (Steinberg, 1963).

Studies with an individual specific monoclonal antibody (Smith and Hildemann, 1986b) show that the cells of two parabiosed fingers of *C. diffusa* do not mix during the several days they are in contact before the cytotoxic reaction begins. Because sponge cells usually migrate constantly, and visual observation of the adhesion between the cells of the surfaces of two parabiosed sponge fingers establishes that they are in intimate contact, the failure of the cells of the two contacting sponges to mix would be surprising if cell movement were active. *In vivo* the suppression of cell movement upon contact with non-self tissue may functionally reduce the potential for parasitic or pathogenic cells to enter the sponge tissue during the early phases of allogeneic contact before the cytotoxic rejection reactions begin.

8-hour reactions

The ability of 48-hour aggregates to carry out the cell killing reaction within 8 hours of allogeneic contact indicates that tissue and cells are able, under properly stimulated conditions, to progress from the primary allogeneic recognition event to the cytotoxic effector activity in only 8 hours. We have also suggested that suppression of aggregation in allogeneic mixtures of cells reflects a signal from primary allogeneic recognition and occurs within one hour of allogeneic cell contact. This signal, which suppresses cell movement, is evident in one hour even in cells that are freshly isolated from intact tissue and that require 36 hours to react with cytotoxicity. If these suggestions are valid, the slowness of the cells to carry out allogeneic recognition *per se* is not the limiting factor in the slower reactions. Rather, the delay seems related to the ability of the immune process to proceed to the cytotoxic effector function in response to allogeneic recognition signals. The striking observation that the gray cells in 48-hour aggregates are rapidly mobilized suggests that the responsiveness of gray cells may be one of the accelerated parameters. The acceleration in the sponges of alloreactivity upon wounding or disruption of the tissue may be conceptually related to the idea in the vertebrate immune system that there are a variety of "danger" signals that make the animal more likely to react to a given antigen as foreign (Matzinger, 1994). In *C. diffusa,* these "danger" signals produced from tissue wounding accelerate cytotoxic reactivity from a time course of 170 hours to one of 8 hours.

We suggest above that the inhibition of aggregation may be a response of cells to the release of a chemokine at the boundary of allogeneic contact. We have demonstrated that gray cells are induced to move to the zone of allogeneic contact, a response analogous to those guided by chemokines generated during the reactions of cells in the vertebrate immune system. In vertebrates, there are many chemokines, each with a variety of functions. Thus, from this model one may suppose that one, or more, chemokines may well act to inhibit general cell movement, preventing the ingress of foreign cells, and to attract gray cells to move through these inhibited cells to the boundary of allogeneic contact.

An overview of the immune reaction of C. diffusa

Superficial contact of *C. diffusa* with non-self tissue or cells is quickly recognized and leads to the production of a signal that suppresses the movement of self cells in the zone of contact. This suppression may reduce the entry of foreign cells into the sponge tissue. If the contact persists, or if contact involves significant tissue damage, an acceleration of the immune process occurs, and gray cells become more responsive to the signal to move to the boundary of allogeneic contact. As the gray cells accumulate at the zone of allogeneic contact, cell killing effector mechanisms are initiated, and all cells in the zone of contact, both foreign and self, are killed. This later response, although harmful to the sponge, presumably eliminates any organism that is attacking or trying to gain entry into the sponge tissue. The state of accelerated reactivity of the sponge immune system may last about three weeks.

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